Beneficial microvascular and anti-inflammatory effects of pravastatin during sepsis involve nitric oxide synthase III

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Background. Sepsis induces microvascular inflammation and production of the vasodilator nitric oxide (NO) via endothelial and inducible nitric oxide synthase (eNOS or NOS III and iNOS or NOS II). Statins are cholesterol-lowering drugs; however, they also attenuate inflammation. This study aimed to determine whether pravastatin protected against sepsis-induced hypotension, loss of vascular tone, and microvascular inflammation via NOS pathways.

Methods. Male Wistar rats (n=18) were anaesthetized and the mesentery prepared for fluorescent intravital microscopy. Animals received either lipopolysaccharide (LPS; n=6); LPS+pravastatin (18 and 3 h before LPS; n=6), or saline as a control, for 4 h.

Results. Mean arterial pressure decreased in LPS-treated animals (P<0.05), but not in those also receiving pravastatin. Acetylcholine-induced relaxation of venules was abolished by LPS but improved by pravastatin. Pravastatin also reduced the increase in nitrite concentration and macromolecular leak from venules induced by LPS (P<0.05). The increased leucocyte adhesion seen in LPS-treated rats was also reduced in those also treated with pravastatin. Immunohistochemical analysis showed that pravastatin increased endothelial cell expression of NOS III during sepsis, but had no effect on LPS-induced up-regulation of NOS II.

Conclusions. Pravastatin improved NOS III-mediated vessel relaxation and exerted anti-inflammatory effects within the microcirculation after LPS administration in rats. Pravastatin therefore appears to have beneficial effects during sepsis, as a result of increased microvascular expression and function of NOS III.

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Sepsis is the major cause of death in intensive care units in the UK. The pathophysiological response to sepsis involves the release of pro-inflammatory cytokines and vasoactive agents such as the vasodilator nitric oxide (NO), which contributes to sepsis-induced decreases in arterial pressure. NO is synthesized from l-arginine in endothelial cells, either by calcium-dependent endothelial nitric oxide synthase (NOS III) or by calcium-independent inducible NOS (NOS II). NO then diffuses into adjacent vascular smooth muscle cells, activating soluble guanylyl cyclase, producing cyclic guanosine 5' monophosphate to induce vessel relaxation via protein kinase G.

Sepsis is often modelled in laboratory studies using animals exposed to a component of Gram-negative bacteria, lipopolysaccharide (LPS), or by faecal peritonitis initiated by caecal ligation and puncture (CLP). LPS can be given at non-lethal doses that allow dissection of mechanisms of inflammation during sepsis, or more correctly, endotoxaemia. In the first few hours, NOS II and NOS III increases and the resulting increase in NO is bactericidal, but can also be detrimental because it induces hypotension and inflammation. After prolonged exposure to LPS (6–48 h), there appears to be a decrease in NOS III, including the sequestration of NOS III into intracellular stores via caveolin-1. Activation of mechanisms that increase NOS III may be beneficial, as previous studies during endotoxaemia demonstrated that decreased capillary blood flow was
reversed by stimulation of NOS III. After CLP, survival also improved in mice over-expressing Akt, which is a signalling molecule for NOS III.

Statins are lipid-lowering drugs which act by inhibiting hepatic conversion of 3-hydroxy-3-methyl-glutaryl-CoA reductase (the rate-controlling enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol). However, statins also have been shown to have anti-inflammatory properties. Statins are hydrophilic (pravastatin and rosuvastatin) or lipophilic (atorvastatin, simvastatin, and fluvastatin) and there is evidence that the different types may have different actions. This study investigated the potential beneficial microvascular effects of pravastatin, which is known to reduce mortality during sepsis in mice.

The hypothesis was that statins reduce microvascular complications during established sepsis via an increase in NOS III.

**Methods**

**Animals and housing**

Male Wistar rats (200–280 g, n=18) were obtained from the Field Laboratories at the University of Sheffield, UK. All experiments were performed ethically in accordance with UK Home Office Law (Project Licence number 40/2809). Animals were held in the animal facilities for at least 1 week before experimental procedures, exposed to light on a 12:12 h cycle, maintained in a humidity- and temperature-controlled environment, maintained on 0.3% sodium, standard, pelleted, commercial diet, and allowed water *ad libitum*.

**Intravital microscopy**

**Surgery**

Rats were anaesthetized with thiopental (induction, 30 mg kg⁻¹ i.p.; maintenance, 40–90 mg kg⁻¹ h⁻¹ i.p.; Intra-Vital Sodium, Rhone-Poulenc Rourer, West Malina, UK), so the mesentery adjoining the ileum could be prepared for fluorescent intravital microscopy. Arterial pressure and heart rate were stable throughout and no pedal reflex could be detected at any time. Fluorescein isothiocyanate (FITC) on celite (10%, A1628, Sigma, UK) was conjugated to bovine serum albumin [66 kDa BSA (98%), A7030, Sigma] as previously described. The right jugular vein was also cannulated for administration of FITC-BSA (i.v. 0.02 g 100 g⁻¹ body wt at T₃₀) and acetylcholine (ACh), along with the left carotid artery for computerized monitoring and continuous recording of mean arterial pressure (MAP) using WINDAQ (DI-400, DATAQ Instruments, Akron, OH, USA). Infusions of LPS (150 µg kg⁻¹ h⁻¹) or the equivalent volume of saline (1 ml kg⁻¹ h⁻¹) were administered via the jugular vein.

**Experimental protocol**

Images were viewed with a ×20 objective (0.30 numerical aperture, Leica, UK), monitored using a CCD camera (TK-C13060B, JVC, UK) with a C-mount adaptor (0.5× HC f. 1/2”) and displayed on a high-resolution monitor (PVM-14N5MDE, Sony, UK) and recorded on to CD-RW using a DataVideo™ CD recorder (VDR-3000, Holdan Ltd, UK) for later off-line computerized image analysis (Capiscope™, KK Technology, UK) with the appropriate frame grabber card capturing at 25 frames per second (Matrox Meteor II, UK). One to two areas of interest containing arterioles (15–40 µm) and veins (25–70 µm) were selected during the equilibration period (30 min) and then recorded for 30 s using epi-illumination every 30 min for the 4 h duration of the experiment (T₀–T₂₄₀).

To measure diameter, Capiscope™ was calibrated with a micrometer specifically designed for the camera and monitor, and the lines were drawn across the vessel to produce a median value in micrometres for each animal at every 30 min time point. The percentage increase in vessel diameter to ACh (1 µg kg⁻¹ i.v.) was calculated at T₂₄₂, after the addition of ACh at T₂₄₀ [T₂₄₂ / (T₂₄₂-T₂₄₀) × 100]]. To measure *macromolecular leak*, Capiscope™ assigned an integer value to the brightness of the interstitial fluorescence based on an arbitrary grey scale ranging from 0 (black) to 244 (white). The fluorescent light intensity thus measured is proportional to the amount of leak. Three small boxes were placed on the screen at random sites adjacent to small venules (<2 µm) to produce a mean of these values in each animal. The same sites were then used for repeated measurements.

**Experimental groups**

Vessels were studied between T₀ and T₂₄₀ min in: (i) saline (n=6), (ii) LPS (infusions commenced at T₀), and (iii) LPS+pravastatin (200 µg kg⁻¹ s.c., 18 and 3 h before T₀) groups (n=18). The 4 h continuous low-dose infusion of LPS (150 µg kg⁻¹ h⁻¹) was based on previous studies. Rats were killed humanely using cervical dislocation at the end of the experiment. At the end of the procedure, cardiac puncture was also performed to obtain serum for measurement of nitrite (NOₓ assay). Mesenteric and lung tissue were removed and placed in formalin for 24 h, followed by undefined abbreviation IMS for up to 1 month before paraffin embedding and sectioning for immunohistochemistry (IHC).

**Immunohistochemistry**

Mesenteric tissue was removed from rats at the end of the experimental protocol and paraffin embedded for IHC with undefined abbreviation DAB in 5 µm sections. Sections were stained with either a polyclonal rabbit anti-human NOS II (1:2400, AbCam) or a rabbit anti-human NOS III (1:800, AbCam) primary antibody, which cross-react with rat tissue, with all experiments performed in one batch, so...
that intensities could be compared. Biotinylated goat anti-rabbit IgG was used as the secondary antibody and either lung (NOS III) or LPS-treated lung (NOS II) used as a positive control. Species-matched non-specific IgG was used as a negative control to ensure that antibody staining was specific.

Five vessel hot spots on each section were randomly selected on each slide (n=6, 5 areas per animal) using a Chalkley grid and analysis was blinded. The diameter of the grid was aligned next to a randomly selected vessel within the grid and following the advice of a consultant histopathologist, endothelial cells along the length of a 25 μm length of vessel graded as either: no staining, which gave an intensity of 0, up to the most intense staining, which had a value of 3. Leucocytes were also counted and graded for intensity of staining (0–3), within the area of the Chalkley grid (~39 μm²).

Serum NOx levels

The StressXpress Nitric Oxide (total) Detection Kit was used according to the manufacturer’s instructions, which quantifies total nitrite colorimetrically using the Griess reaction. Frozen serum was defrosted, diluted 1:10, and analysed in duplicate. The absorbance in each well between 540 and 570 nm was determined using a plate reader. The average net absorbance for each standard and sample was calculated by subtracting the average zero standard absorbance from the average absorbance for each standard and sample. This allowed nitrite levels to be calculated (NOx), as an indicator of total NO production.

Statistical analysis

Arterial pressure, vessel diameters, and macromolecular leak are expressed as percentage changes from values at T0 and compared with the LPS group unless otherwise stated. All data are presented as mean (SD). Intravital microscopy (IVM) data were analysed using two-way ANOVA, followed by the Bonferroni post hoc test to identify points of significance, apart from ACh responses and nitrite/nitrate data, which were assessed using one-way ANOVA followed by Dunnett’s test. IHC data were analysed using two-way ANOVA, followed by the Bonferroni test to identify points of significance. All statistical analyses were performed using Graphpad Prism version 5.0. Data were considered statistically significant at P<0.05.

Results

Arterial pressure

LPS produced a decrease in MAP (mm Hg) at all time points, as demonstrated between 0 [124 (std 11.0)] and 240 min [63.9 (11.9)] (P<0.05) (Fig. 1). Pravastatin reduced LPS-mediated hypotension as there was no significant decrease in MAP [0 min, 129.4 (5.1); 240 min, 88.3 (8.9)], but this did not reach significance when compared with the LPS group. In the controls, there was also no significant decrease in MAP [0 min, 125.6 (10.9); 240 min, 114.4 (4.2)]. Heart rate increased in the presence of LPS [0 min, 375 (20.1); 240 min, 480 (25.7)] (P<0.05), but not in controls [0 min, 380 (2.0); 240 min, 390 (17.3)] or in the LPS+pravastatin-treated group [0 min, 370 (22.1); 240 min, 372 (26.0)].

Microvascular inflammation

Macromolecular leak

LPS caused an increase in interstitial fluorescence adjacent to post-capillary venules over 4 h of the experiment [60 min, 281.1 (98.9); 120 min, 339.7 (116.1); 180 min, 324.6 (132.2); 240 min, 293.6 (104.4)] (P<0.05), due to macromolecular leak of FITC-BSA (Fig. 2A). Controls exhibited no leak at these time points [60 min, -16.5 (15.6); 120 min, -14.5 (24.0); 180 min, 24.5 (41.1); 240 min, 89.8 (100.8)]. Pravastatin reduced LPS-mediated leak at 180 [72.6 (43.5)] and 240 min [52.3 (51.4)].

Leucocyte–endothelial interactions

LPS caused an increase in the numbers of adherent leucocytes at all time points, as demonstrated between 0 [0.7 (0.4)] and 240 min [8.0 (1.6)] (P<0.05) (Fig. 2B). Pravastatin decreased LPS-induced adhesion [0 min, 1.7 (0.7); 240 min, 1.2 (0.8)] and similarly, no significant increases were observed in controls [0 min, 0.3 (0.3); 240 min, 0.5 (0.5)].

NO endothelial-dependent relaxation

There were no significant changes in the diameters of arterioles or venules throughout the experiment in any group (Fig. 3). However, ACh caused dilation of venules [17.8 (3.8)%] in the control group (P<0.05), and this was
Expression of NOS II and NOS III

Endothelial expression

NOS III expression was similar in both controls and LPS-treated tissue (Figs 4 and 5). However, in the presence of LPS, pravastatin caused greater expression of NOS III than that observed in controls ($P<0.05$). LPS increased expression of inflammatory NOS II compared with controls, but increased NOS II expression was retained in the presence of pravastatin ($P<0.05$).

Leucocyte expression

LPS decreased the numbers of leucocytes that expressed NOS II and NOS III compared with controls and this was partially restored to control levels by pravastatin, but did not reach significance (NOS III, $P=0.05$; NOS II, $P=0.35$) (Fig. 6). However, no significant differences were detected between the intensities with either isoform.

Serum (NO$_X$) concentration

LPS increased serum concentration of NO$_X$ [controls, 37.2 (0.9) µM; LPS, 57.3 (2.8) µM] ($P=0.002$). Pravastatin then reduced the NO$_X$ concentration in the presence of LPS [LPS + pravastatin, 40.0 (7.7) µM] ($P=0.052$).

abolished after 4 h LPS [4.4 (7.4)%%]. In the presence of LPS, vasodilation to ACh was restored by pravastatin [24.1 (7.9)%%].
Discussion

The rationale for performing this study was that the homeostatic balance of NOS II and NOS III is important during sepsis, contributing to cardiovascular complications and agents such as statins, which alter the balance of NOS II: NOS III may have protective microvascular effects. In summary, these data showed that the widely prescribed hydrophilic statin, pravastatin, reduced macromolecular leak and leucocyte adhesion within the rat mesentery 2–4 h after induction of endotoxaemia with LPS. It thus demonstrated potentially beneficial anti-inflammatory effects within the microcirculation. Pravastatin also restored vessel relaxation to ACh in LPS-treated vessels. Concurrently, in LPS-treated tissue, pravastatin increased endothelial cell expression of NOS III. Taken together, pravastatin appears to exert beneficial effects on vessel tone and possibly arterial pressure, via an NOS III pathway. Previous studies have reported that pravastatin increases NOS III in endothelial cells and our findings during endotoxaemia are in agreement with this. Pravastatin also reduced hind limb ischaemia in rats and restored blood flow by an NOS III mechanism, but there is presently a lack of relevant mechanistic studies during sepsis with which to compare this particular statin.

If we consider endotoxaemia alone, previous studies have demonstrated that LPS causes hypotension, initially due to increased expression and function of NOS III, but this mechanism may become less important, or even down-regulated, as sepsis progresses. In agreement with this, our IHC studies reported that LPS did not alter endothelial NOS III expression, but did abolish NOS III function, namely vessel relaxation to ACh. We therefore propose that in our model, there may be unaffected expression but reduced function due to alteration of pathways either up- or downstream of NOS III. There is some evidence that the latter may be due to changes in Akt phosphorylation during sepsis. Lack of vessel responsiveness to ACh could also be the result of free radical production, but this is not something we could elucidate from the present study.

We demonstrated that pravastatin both increased relaxation to ACh and expression of NOS II during LPS administration. This is perhaps not surprising, as up-regulation of NOS III by statins may occur due to inhibition of the GTPase Rho, which depends upon geranylation by the isoprenoid geranylgeranyl-PP (part of the HMCoA reductase pathway). The interaction between Rho and NO is the focus of our ongoing research. However, in this study, despite endothelial NOS III expression being unaffected by endotoxaemia alone, our results indicate that by increasing NOS III beyond LPS and control concentrations pravastatin improves vessel responses to ACh. As pathways

Fig 5 Mesenteric blood vessels from controls (A), LPS-treated (B), LPS+pravastatin-treated (C), and negative control (D) stained for expression of NOS III. The intensity of endothelial cell NOS III staining lining the blood vessels was similar in controls (A) and LPS-treated (B) animals, but increased ('black' areas) in the presence of LPS+pravastatin (C). No staining was identified in negative controls.
downstream are malfunctioning, greater levels of NOS III may be required for function of the vessel, thus pravastatin has a beneficial action. In agreement with our findings for endothelial cell expression, simvastatin and lovastatin have been shown to increase NOS III expression in cultured human saphenous vein endothelial cells. Similarly, rosuvastatin up-regulated expression of mRNA for NOS III and protected from cerebral ischaemia in mice. Interestingly, the effects on macromolecular leak occurring between 2 and 4 h when NOS III is up-regulated, despite pravastatin having no apparent effect on this isoform. Hence, further functional studies are probably required using specific and non-specific NOS inhibitors.

Using IHC, we also determined that LPS also caused up-regulation of NOS III in the endothelium, which is a known phenomenon and in agreement with previous literature. However, we did not observe any reduction in endothelial cell expression of NOS II with pravastatin. This is in disagreement with previous studies, as chronic administration of simvastatin (10 mg kg\(^{-1}\)) prevented aortic ring relaxation to LPS-induced NOS II. Other studies have demonstrated that simvastatin decreases expression of NOS II in embryonic cardiac myoblasts in response to TNF-\(\alpha\) and IL-1. We therefore postulate that hydrophilic statins, such as pravastatin, may have differential effects on NOS II (and NOS III) compared with lipophilic statins, such as simvastatin, and this will form the basis of our future investigations. Indeed, when several experiments have demonstrated that pravastatin reduced serum levels of NO\(_x\). However, LPS increased NOS II expression remained unaffected by pravastatin. Surprisingly, however, serum NO\(_x\) was reduced by pravastatin. Therefore, it could be proposed that the mesentery is behaving differently to other organs. Nevertheless, in our model of sepsis, at 4 h, pravastatin did not appear to be exerting anti-inflammatory effects in the mesentery via a reduction in NOS II.

We observed decreased leucocyte adhesion induced between 2 and 4 h of sepsis. Leucocyte adhesion is also indicative of inflammation as it is a prerequisite to migration into tissues, where leucocytes are able to release cytokine and oxygen free radicals responsible for tissue damage and multiple organ failure during sepsis. Pravastatin did not alter the numbers of leucocytes expressing NOS III. On the contrary, LPS decreased NOS II, but was restored by pravastatin. This unusual decrease in leucocyte NOS II during inflammation has previously been reported in a number of studies. Pravastatin responses may be in accordance with an anti-inflammatory effect, there are different NOS mechanisms occurring in leucocytes, compared with endothelial cells that are beyond the scope of the current manuscript and require more detailed exploration by leucocyte biologists.

This study also reports that pravastatin reduced LPS-induced leakage of FITC-BSA from post-capillary venules; hence, we have identified an important anti-inflammatory effect that will contribute to reduced oedema and improvement of blood volume: a desirable property for recommending the use of statins in an intensive care setting. Interestingly, the effects on macromolecular leak occurred between 2 and 4 h when NOS III is up-regulated, despite pravastatin having no apparent effect on this isoform. Hence, further functional studies are probably required using specific and non-specific NOS inhibitors in vivo.
Statins are compared for their effects on macrophage release of NO via NOS II (mediated by effects on NF-kappa-B and STAT1 upstream of NOS II), there is a potency order of lovastatin > atorvastatin > fluvastatin > pravastatin. Pravastatin also causes a more potent effect on NOS III expression compared with atorvastatin in rat lung. Therefore, it may be that pravastatin exerts its beneficial effects via NOS III, whereas other statins have more potent effects on NOS II and it would useful to investigate other statins using our experimental model.

Despite our finding that pravastatin reduces macromolecular leak and leucocyte adhesion in the rat mesenteric microcirculation during sepsis, these data should be interpreted cautiously. First, reduced inflammation does not always result in reduced mortality, and mortality studies are required before we can conclude that pravastatin is beneficial during sepsis. Thus far, Merx and colleagues have reported that simvastatin, atorvastatin, and pravastatin, but not fluvastatin, reduced mortality during CLP in rats. High levels of NOS III activation were also reported 20 h after CLP, highlighting potential differences in NOS III expression depending on the model used and the timing of NOS measurements. In addition, LPS is simulating septic conditions in a rat model and findings need to be repeated in both patients and the intensive care setting. It is important to note that our animals were anaesthetized and anaesthesia itself may alter microvascular responses to LPS.

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